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Presence of One Linear and One Circular Chromosome in the *Agrobacterium tumefaciens* C58 Genome

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Analysis of the entire *Agrobacterium tumefaciens* C58 genome by pulsed-field gel electrophoresis (PFGE) reveals four replicons: two large molecules of 3,000 and 2,100 kb, the 450-kb cryptic plasmid, and the 200-kb Ti plasmid. Digestion by *Pac*I or *Swa*I generated 12 or 14 fragments, respectively. The two megabase-sized replicons, used as probes, hybridize with different restriction fragments, showing that these replicons are two independent genetic entities. A 16S rRNA probe and genes encoding functions essential to the metabolism of the organism were found to hybridize with both replicons, suggesting their chromosomal nature. In PFGE, megabase-sized circular DNA does not enter the gel. The 2.1-Mb chromosome always generated an intense band, while the 3-Mb band was barely visible. After linearization of the DNA by X-irradiation, the intensity of the 3-Mb band increased while that of the 2.1-Mb remained constant. This suggests that the 3-Mb chromosome is circular and that the 2.1-Mb chromosome is linear. To confirm this hypothesis, genomic DNA, trapped in an agarose plug, was first submitted to PFGE to remove any linear DNA present. The plug was then recovered, and the remaining DNA was digested with either *Pac*I or *Swa*I and then separated by PFGE. The fragments corresponding to the small chromosome were found to be absent, while those corresponding to the circular replicon remained, further proof of the linear nature of the 2.1-Mb chromosome.

Classically, bacteria were thought to possess a single, circular chromosome (17). This belief was shattered by the recent report of the existence of two circular chromosomes in *Rhodobacter sphaeroides* (29, 30) and of a single linear chromosome in *Borrelia burgdorferi* (4, 9), in *Rhodococcus fascians* (6), and in *Streptomyces lividans* (19). Further examples of complex genomic organization are *Brucella melitensis*, which possesses two chromosomes (22), and *Rhizobium meliloti*, which possesses one chromosome and two megaplasmids (28).

So far, all the organisms with multiple megareplicons are members of the alpha group of the class *Proteobacteria*, as defined by their 16S rRNA sequences (36). Moreover, the 16S rRNA gene sequences reveal close relationships between some members of this group: *Brucella*, *Agrobacterium*, and *Rhizobium* species (24, 37). While investigating the genomic organization of the group further, we have found that *Agrobacterium tumefaciens* C58, a strain known to contain two large plasmids (15, 31), in fact possesses an even more complex genome, with four replicons, including two chromosomes of different topology.

(Partial results of this work were presented at the First International Symposium Mapping and Sequencing of Small Genomes [2].)

MATERIALS AND METHODS

Strains and growth conditions. *R. meliloti* 2011 and *A. tumefaciens* C58 were gifts from Maria Fernandez and Xavier Nesmes (UA 697 and URA CNRS 1454, Ecologie microbienne du sol, Université Claude Bernard Lyon 1, Villeurbanne, France). *R. meliloti* 2011 was grown at 28°C on yeast mannitol agar, and *A. tumefaciens* C58 was grown in yeast extract-peptone medium at 28°C. *B. melitensis* 16M is from our laboratory collection and was grown as previously described (1). Plasmid pCD523 containing the chromosomal virulence *chvA* and *chvB* genes (8) was a gift from X. Nesmes. Plasmids pCD1, containing the *A. tumefaciens* C58 genes encoding phospho-mannose isomerase activity, and pYDS100, containing the genes encoding enzymes involved in proline metabolism, were gifts from Yves Dessaux (Institut des Sciences Végétales, Centre National de la Recherche Scientifique, Gif sur Yvette Cedex, France).

PCR amplification. Primers for the amplification of most eubacterial 16S rRNAs have been described elsewhere (11, 34). PCR was carried out with 100 to 500 ng of *A. tumefaciens* C58 DNA as a template, 50 pmol of primers fD1-rP2 (34) or PC5-P3mod (11), 100 μM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, and 2 U of *Taq* DNA polymerase (Promega), in the buffer supplied by the manufacturer. Thirty cycles, each corresponding to 95°C for 45 s, 35°C for 1 min, and 72°C for 2 min, were realized. The combinations of fD1-rP2 or PC5-P3mod yielded fragments of 1,100 or 700 bp, respectively.

Preparation of high-molecular-weight genomic DNAs and endonuclease digestion. Intact genomic DNAs were prepared in agarose plugs as previously described (1). *A. tumefaciens* C58 DNA was digested with restriction endonuclease *Pac*I (New England Biolabs) or *Swa*I (Boehringer) by following the recommendations of Sobral et al. (28).

PFGE of intact and digested DNAs. Pulsed-field gel electrophoresis (PFGE) was performed in a contour-clamped homogeneous electric field (CHEF) apparatus, either CHEF-DRII (Bio-Rad) or Gene Navigator (Pharmacia), in 0.5× TBE (21). Undigested DNAs were electrophoresed through a 0.6% agarose gel for 48 h with either a pulse ramp of 1.30 to 5 min at 150 V with the CHEF-DRII or a ramp of 5 to 20 min at 80 V with the Gene Navigator. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosomes (Bio-Rad) served as size markers. Separation of *Pac*I and *Swa*I fragments was obtained in a 1% agarose gel with two pulse ramps, 10 to 30 s for 15 h and 60 to 150 s for 30 h at 150 V, with *S. cerevisiae* chromo-

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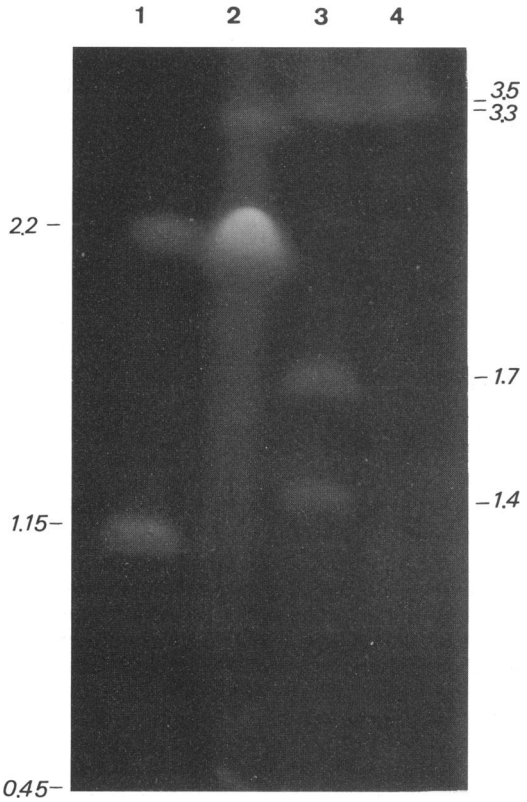

FIG. 1. PFGE of total genomic DNA: *B. melitensis* (lane 1), *A. tumefaciens* C58 (lane 2), *R. meliloti* (lane 3), and *S. pombe* (lane 4). Sizes of the markers are given in megabase pairs. The pulse ramp was 90 to 300 s for 45 h at 150 V.

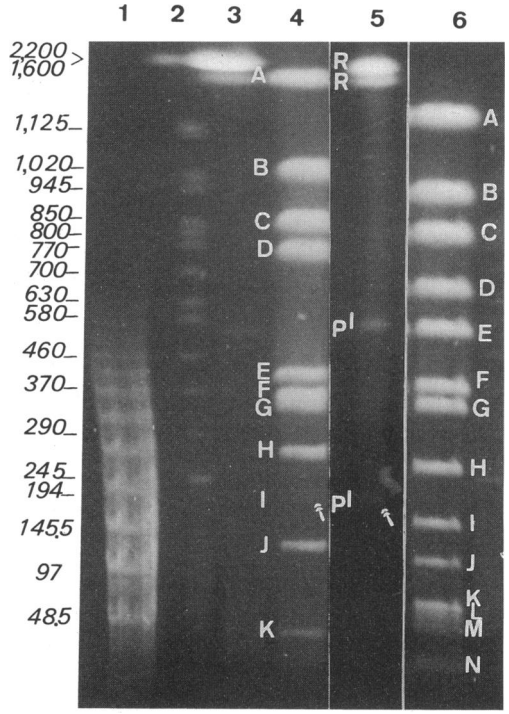

FIG. 2. PFGE of restricted *A. tumefaciens* C58 DNA: *Pac*I digestion (lane 4), *Swa*I digestion (lane 6), undigested C58 genomic DNA (lanes 3 and 5), lambda concatemers (lane 1), and *S. cerevisiae* chromosomes (lane 2). The locations of *Pac*I and *Swa*I fragments are shown on the left of lane 4 and on the right of lane 6, respectively (*Pac*I-L, the smallest fragment, ran out the gel; see Fig. 5). The locations of megareplicons (R) and cryptic and Ti plasmids (pl) are shown on the left of lane 5. The *Pac*I-I band and Ti plasmid are shown by white arrows. Sizes of the markers, in kilobase pairs, are shown on the left. Pulse ramps were 10 to 30 s for 15 h and 60 to 150 s for 30 h at 150 V; inversion of the 2.1- and 3-Mb bands was seen with these conditions.

somes and multimers of phage lambda (prepared as previously described [1]) as molecular size standards.

DNA hybridizations. Southern blots of PFGE agarose gels were prepared on nylon membranes (Boehringer) by vacuum blotting with 0.4 M NaOH as the eluant. Probes were labelled by a nonisotopic method (digoxigenin DNA labeling kit [Boehringer]) according to the manufacturer's instructions. Nondigested replicons were purified by Gene Clean II (Bio 101) after separation in low-melting-temperature agarose (SeaPlaque) and then labelled.

X-irradiation studies. Plugs containing *A. tumefaciens* C58 DNA were exposed to increasing dosages of X rays from a linear accelerator (Saturne 43 from General Electric) with an energy of 6 MeV and delivering 400 rads/min.

RESULTS AND DISCUSSION

***A. tumefaciens* C58 contains multiple replicons.** The existence of megareplicons has been shown by the use of PFGE in *R. sphaeroides* (29, 30), in *R. meliloti* (28), and in *B. melitensis* (22). Using PFGE with the CHEF system, we examined the total genomic DNA of *A. tumefaciens* C58, which contains large cryptic plasmid pAtC58 and the tumor-inducing plasmid pTi (15, 31). The different replicons of the C58 genome were resolved by PFGE (Fig. 1). Using a combination of long pulse times, low voltage, low agarose concentration, and a greater concentration of DNA (five times greater than usual), two high-molecular-weight bands with apparent sizes of 3 and 2.1 Mb and a smaller band corresponding to the 450-kb pAtC58 were separated (Fig. 1, lane 2). The 2.1-Mb band was always very thick and intense, whereas the 3-Mb band was faint. The 2.2- and 1.15-Mb *B. melitensis* chromosomes (22) (Fig. 1, lane 1) and the 3.3-, 1.7-, and 1.4-Mb *R. meliloti* chromosome and megaplasmids (28) (Fig. 1, lane 3) generated bands of similar intensity (thus showing equimolarity). These DNA molecules, together with the smallest, 3.5-Mb *S. pombe* chromosome (Fig. 1, lane 4), also served as size markers. The two largest *S. pombe* chromosomes are too big to be efficiently separated by the pulse times used. Similarly, the 200-kb pTi of *A. tumefaciens* C58 is too small and ran out of the gel. The Ti plasmid can be seen if shorter pulses are employed (Fig. 2, lane 5).

The two megabase-sized replicons are separate genetic entities. The presence of two megabase-sized bands suggested that *A. tumefaciens* C58 has a complex genomic organization. To confirm that the two bands were two separate genetic entities, we employed methods which we have previously used for *B. melitensis* (22). *Agrobacterium* spp. DNA possesses a high G+C content (16); therefore, restriction endonucleases with A+T-rich recognition sites, such as *Pac*I (5′-TTAAT TAA-3′) and *Swa*I (5′-ATTTAAAT-3′), cut these genomes infrequently (28). Digestion of C58 DNA with *Pac*I or *Swa*I generated 12 or 14 fragments (Fig. 2, lanes 4 and 6). The sizes of the bands, estimated relative to *S. cerevisiae* linear chromosomes and lambda concatemer molecular weight markers, are

TABLE 1. Sizes of the *Pac*I- and *Swa*I-generated restriction fragments from *A. tumefaciens* C58

Fragment	Size (kb) generated by:	
	*Pac*I	*Swa*I
A	1,420	1,125
B	965	895
C	790	770
D	710	640
E	385	515
F	350	365
G	330	325
H	250	245
I	200	165
J	140	120
K	35	75
L	10	65
M		55
N		20
Total	5,585	5,380

shown in Table 1. Calculation of the genome size from the sums of the restriction fragments shows a 200-kb discrepancy between the *Pac*I (5,585 kb) and *Swa*I (5,380 kb) digests. However, the *Pac*I band I (200 kb), (Fig. 2, lane 4) migrated at the same distance as the smallest band of the nondigested total genome (Fig. 2, lane 5). The *Pac*I I band is faint compared to the *Pac*I J band and as such could be interpreted as a partial digestion; however, in this case, it would give other faint bands. We suggest that both bands were linearized forms of the 200-kb plasmid generated either by cleavage at a unique *Pac*I site or, more likely, by a random break during DNA preparation or incubation with *Pac*I which gave an intense background smear. This plasmid was not digested with *Swa*I and therefore did not enter the gel. Measurements of the chromosome of *A. tumefaciens* by electron microscopy estimate it to be larger than 5 Mb (5) or at least 5.5 Mb from the renaturation rate (12). The results of our restriction fragment analysis show that the genome of strain C58 is approximately 5,600 kb in length, consistent with the previous estimations.

To determine from which replicon the individual restriction fragments originated, the 3-Mb, 2.1-Mb, and 450-kb bands were purified from low-melting-temperature agarose and labelled by a nonisotopic method. Southern blots of PFGE agarose gels were hybridized with the undigested replicons as probes. Unfortunately, we did not obtain enough material in the 200-kb band to make a good probe. Figure 3 and Table 2 show hybridization of probes made from the 3-Mb, 2.1-Mb, and 450-kb bands to *Pac*I and *Swa*I digests. The 3-Mb probe hybridized with *Pac*I fragments A, C, E, and H and *Swa*I fragments A, B, G, H, J, K, and L (Fig. 3A and B, lanes 2). The 2.1-Mb probe hybridized *Pac*I-B, -D, and -F and *Swa*I-C, -D, -E, -K, -L, and -M (Fig. 3A and B, lanes 3). The 450-kb probe hybridized with *Pac*I fragments G and J and *Swa*I fragments F and I (Fig. 3A and B, lanes 1). The specificity of hybridizations of the different probes was deduced from the intensities of the signals. Besides the strongly hybridized fragments, others were also labelled, although more faintly, and were considered nonspecific. Gel-purified genomic fragment probes usually contain small amounts of randomly broken DNA from the rest of the genome and therefore hybridize faintly to all fragments (1). Hybridizations to some small fragments were difficult to interpret. None of the three replicons hybridized with *Pac*I-I, -K, and -L nor with *Swa*I-N. This can be explained for *Pac*I-I,

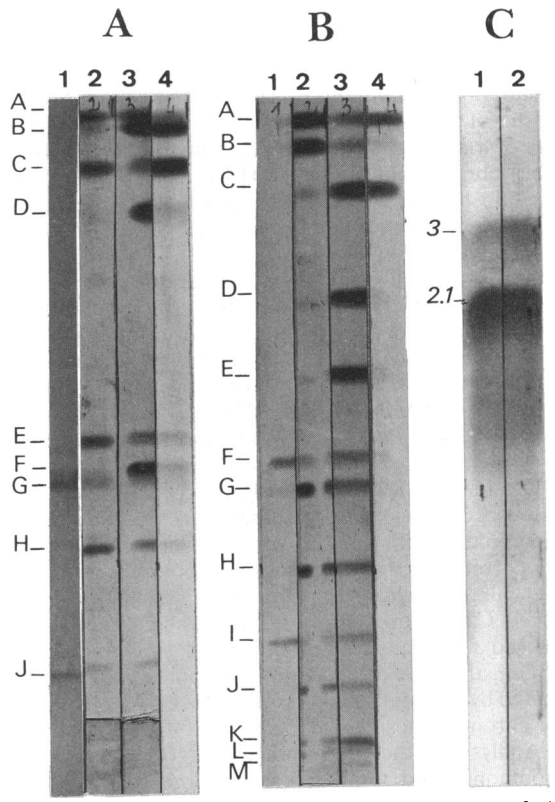

FIG. 3. Evaluation of the genetic content of the *A. tumefaciens* C58 megareplicons. Hybridization with the 450-kb plasmid probe (lane 1), the 3-Mb replicon probe (lane 2), the 2.1-Mb replicon probe (lane 3), and the 16S rRNA probe (lane 4) on *Pac*I-digested DNA (A) or *Swa*I-digested DNA (B). Fragments are labelled on the left of each panel. (C) Hybridization with the 16S rRNA probes: 700-bp (lane 1) and 1,100-bp (lane 2) PCR products on undigested DNAs, 3- and 2.1-Mb replicons, labelled on the left.

which corresponds to the linearized 200-kb pTi. For *Pac*I-K and -L and *Swa*I-N, the lack of hybridization may be due to poor transfer of fragments that were randomly broken during depurination and were then too small to transfer efficiently to the nylon membrane. Both the 3- and 2.1-Mb probes hybridized with *Swa*I-K and -L. *Swa*I-K appears to be a doublet: it was more strongly hybridized with the 2.1-Mb probe, suggest-

TABLE 2. Hybridization of the *Pac*I and *Swa*I fragments by different probes

Probe[a]	Hybridized fragment(s)	
	*Pac*I	*Swa*I
3 Mb	A, C, E, H	A, B, G, H, J, K, L
2.1 Mb	B, D, F	C, D, E, K, L, M
450 kb	G, J	F, I
pCD523	C	A
pCD1	F	D
pYDS100	B	C
16S rRNA	B, C	A, C

[a] Probes correspond to the 3-Mb, the 2.1-Mb, and the 450-kb replicons; the *A. tumefaciens* chromosomal virulence genes *chvA* and *chvB* (pCD523); the genes encoding phospho-mannose isomerase activity (pCD1); the genes encoding enzymes involved in proline metabolism (pYDS100); and the 16S rRNA genes (obtained by PCR amplification).

ing that this band was more likely derived from this replicon, but in another experiment, *Swa*I-K was also assigned to the bigger replicon (see below the results of premigrated DNA [Fig. 5, lane 3]). Examination of the *Swa*I-K band on the gel showed that it was more heavily stained by ethidium bromide than was *Swa*I-L (Fig. 2, lane 6) and thus is probably derived from both chromosomes. For *Swa*I-L, hybridization by both probes could be due to the presence of insertion sequences (32), as the results of premigrated DNA assigned *Swa*I-L to the bigger replicon (see below, Fig. 5, lane 3).

The three probes hybridized with different restriction fragments, and we can conclude from this experiment that the corresponding replicons are different genetic entities. The sum of the fragment sizes hybridized with the different probes represents 2,845 kb (*Pac*I) or 2,850 kb (*Swa*I) with the 3-Mb probe, 2,025 kb (*Pac*I) or 2,120 kb (*Swa*I) with the 2.1-Mb probe, and 470 kb (*Pac*I) or 530 kb (*Swa*I) with the 450-kb probe.

Thus, besides the two previously described plasmids, two megabase-sized replicons which together represent almost the whole genomic content of *A. tumefaciens* C58 can be seen. This does not correlate with the findings of Hooykaas et al. (14) or Miller et al. (23), from which a circular map of the *A. tumefaciens* C58 unique chromosome was constructed. These authors used a chromosomal mobilizing plasmid to construct a genetic map, and the existence of two chromosomes could have been missed by this method. Direct evidence of the existence of multiple chromosomes was made possible with the advent of PFGE. Analysis of the total undigested genome of *R. sphaeroides* demonstrated for the first time the presence of two large physical DNA structures (29). Genetic evidence for the existence of two chromosomal linkage groups was shown later with the development of a genetic Hfr system in *R. sphaeroides* (30). The 12 and 14 fragments generated by *Pac*I and *Swa*I are the first steps in the construction of a physical map of the *A. tumefaciens* C58 genome.

Are both megabase-sized replicons chromosomes? The existence of multiple chromosomes (as opposed to one chromosome and one, or several, megaplasmid[s]) is still controversial (17). We reason that the presence of genes encoding functions essential to the metabolism of the organism is indicative of a chromosome (22, 29, 30). Probes made from both 16S rRNA PCR products hybridized with the two nondigested megabase-sized replicons (Fig. 3C). The 700-bp 16S rRNA probe was found to hybridize to the *Pac*I C and *Swa*I A fragments which originate from the 3-Mb replicon and to the *Pac*I B and *Swa*I C fragments which originate from the 2.1-Mb replicon (Fig. 3A and B, lanes 4, and Table 2). Plasmid pCD523 contains *A. tumefaciens* chromosomal virulence genes *chvA* and *chvB*, which encode proteins required for the attachment of *A. tumefaciens* to plant cells (8). A probe made with this plasmid hybridized with the *Pac*I C and *Swa*I A fragments, which both originate from the 3-Mb replicon (Table 2). Cloned genes encoding proteins involved in the phospho-mannose isomerase activity and the metabolism of proline (pCD1 and pYDS100) hybridized with fragments derived from the 2.1-Mb replicon (*Pac*I-F or *Swa*I-D for pCD1 and *Pac*I-B or *Swa*I-C for pYDS100; Table 2). The presence of metabolically essential genes on both replicons allows us to argue that both are chromosomes.

The 2.1-Mb replicon is linear. The difference seen between the intensities of the 3- and 2.1-Mb bands suggested that the 2.1-Mb band was present in more copies than the 3-Mb band. This, however, was not consistent with the intensity of the bands generated by *Pac*I or *Swa*I digests, which suggested equal stoichiometry. A second explanation was that the 2.1-Mb replicon is linear and the 3-Mb replicon is circular. Linear molecules of DNA up to several megabases in length are separated, whereas open circular DNA molecules larger than 15 kb fail to migrate in PFGE and remain trapped in the well (20). To determine whether the replicons were linear or circular, we used X-irradiation, which, like γ rays, produces random double-stranded breaks in DNA (33), and examined the behavior of the different molecules in PFGE. Figure 4 shows total genomic DNA of *A. tumefaciens* C58 exposed to 1, 4, or 16 krads and then subjected to CHEF electrophoresis. When DNA was not irradiated, the 2.1-Mb band was thick and intense whereas the 3-Mb, 450-kb, and 200-kb bands were not visible (lane 2). At 1 krad, the intensity of the 3-Mb band increased, as did that of the 450- and 200-kb bands (lane 3). Exposure to 4 krads produced a further increase in the intensity of these bands, whereas the intensity of the 2.1-Mb band began to decrease (lane 4). Exposure to 16 krads resulted in a smear of smaller fragments (lane 5), indicating multiple random double-stranded breaks per molecule. This experiment shows that two different species of molecule exist in the genome of *A. tumefaciens* C58: first, a thick intense 2.1-Mb band which behaves like the linear chromosomes of *S. cerevisiae*, the 1-Mb linear chromosome of *B. burgdorferi* (4, 9), and the 4-Mb linear chromosome of *R. fascians* (6), since it entered the gel and migrated like a linear duplex DNA, and second, three replicons of 3 Mb, 450 kb, and 200 kb which do not enter the PFGE gel unless irradiated. The small amounts of DNA seen to migrate from the nonirradiated preparations are probably molecules randomly broken during preparation, as was reported for *R. meliloti* (28). We used the fact that only a linear molecule enters the gel when CHEF is applied, whereas large circular forms remain trapped in the agarose blocks to further confirm the linearity of the 2.1-Mb replicon. Two blocks were first submitted to PFGE, then removed from the well, rinsed abundantly to remove the electrophoresis buffer, and digested with either *Pac*I or *Swa*I. As a control, two blocks

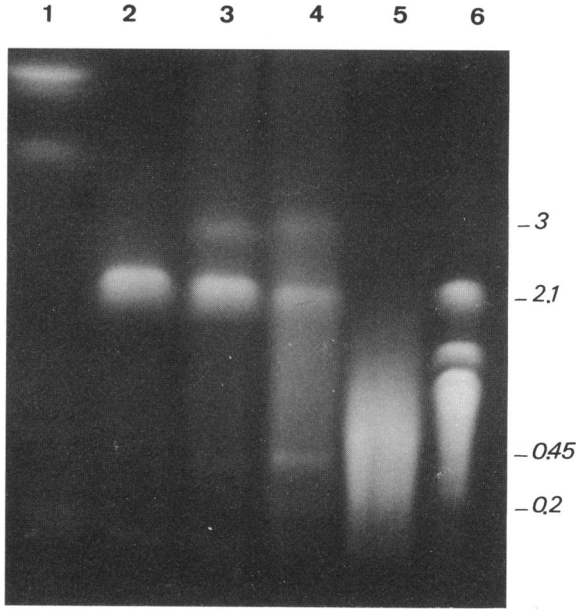

FIG. 4. X-irradiation of total genomic C58 DNA: 1 krad (lane 3), 4 krads (lane 4), and 16 krads (lane 5). Lane 2, nonirradiated control DNA; lane 1, *S. pombe*; lane 6, *S. cerevisiae*. Sizes of the four *A. tumefaciens* C58 replicons are given in megabase pairs.

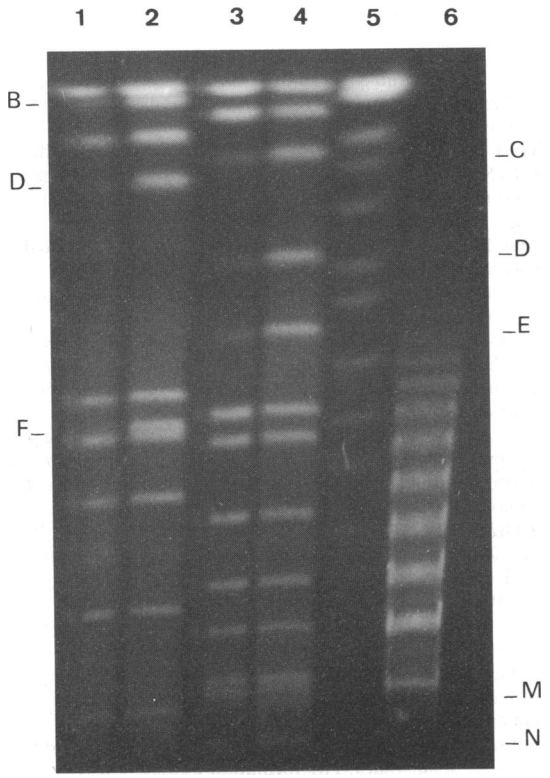

FIG. 5. PFGE of *A. tumefaciens* C58 DNA premigrated and then digested with *Pac*I (lane 1) or *Swa*I (lane 3) and of controls (no premigration but same digestions, lanes 2 and 4). *Pac*I-B, -D, and -F and *Swa*I-C, -D, -E, -M, and -N disappear (labelled on the left and right sides, respectively). Lane 5, *S. cerevisiae*; lane 6, lambda concatemers.

not premigrated were digested in the same conditions and loaded in parallel on the gel. Results of this experiment are shown in Fig. 5. As expected, *Pac*I fragments B, D, and F (lane 1) as well as *Swa*I fragments C, D, E, M, and N (lane 3) disappeared or were of greatly reduced intensity when genomic DNA was premigrated. These fragments are all from the 2.1-Mb chromosome.

We can therefore conclude that the genomic structure of *A. tumefaciens* C58 is unusual, since it is composed of two permanent megareplicons (presumably chromosomes) of different topology, one circular and one linear. The coexistence of linear and circular DNA molecules in the same bacterial cell has already been described for *B. burgdorferi* (3, 9). In this species there is one large linear chromosome together with smaller linear plasmids (or minichromosomes, as suggested by those authors) and circular plasmids. As far as we know, the coexistence of linear and circular chromosomes in the same cell is unprecedented. This raises questions about the mechanisms of replication of the linear chromosome (atypical origin of replication, as suggested for *B. burgdorferi* [25, 26]) and partitioning of the different replicons during cellular division. Do these different molecules have different or equivalent mechanisms of segregation in the daughter cells? The structure of a linear replicon seems quite different from that of a circular one. In *B. burgdorferi*, linear plasmids have telomeric structures (13) and the properties of the ends of the linear chromosome are consistent with the presence of telomere-like structures (7, 27). As these structures stabilize the ends of linear DNA molecules, they should be present on the *A. tumefaciens* C58 2.1-Mb chromosome. Further investigations are needed to demonstrate their presence.

Finally, these results raise the question of the taxonomic value of the character "complex genome" (as defined by the possession of several independent, segregationally stable megareplicons, chromosomes or megaplasmids, at the exclusion of classic plasmids which are transient and nonessential). This characteristic is sufficiently unusual in the eubacterial domain to be considered autapomorphic (35) if it is found in related taxons. Although unique circular chromosomes are the most common in bacterial genomes (17), it is now well established that complex genomic organization with multiple chromosomes does exist in a few bacterial species. This is the case for *R. sphaeroides* (29, 30), *R. meliloti* (28), *B. melitensis* (22), and *A. tumefaciens* (this study). Continued investigation will probably reveal other organisms showing this feature. It would be wise to look at the number of large replicons by PFGE analysis and perhaps conduct an experiment of γ- or X-irradiation before constructing a physical map. We first published a physical map of a single chromosome for *B. melitensis* and later found that the genome contained in fact two chromosomes (1, 22). It should be added that it seems easier to look for this feature once the dogma of a unique circular bacterial chromosome has been broken.

Until now, all bacteria for which a complex genomic structure has been demonstrated belong exclusively to the alpha subdivision of the class *Proteobacteria* in the Woese classification based on the 16S rRNA sequences (36). However, the same subgroup also contains bacteria with a single chromosome, such as *Bradyrhizobium japonicum* (18) and *Rhodobacter capsulatus* (10). A combination of two techniques was used to construct a correlated physical and genetic map of *B. japonicum* in which the linkage of the restriction fragments was compatible with a circular chromosome. This was not the case of *R. sphaeroides* (29), in which all restriction fragments could not be joined in a contiguous set. Moreover, in the phylogenetic tree of the family *Rhizobiaceae* constructed by Yanagi and Yamasoto (37), *B. japonicum* was far remote from the other species and should be excluded from this family. The single chromosome of *R. capsulatus* (10) is deduced from the construction of a physical map by two methods: overlapping of large restriction fragments and grouping cosmids of a genomic library into contigs. There is, as far as we know, no direct observation of a unique band in PFGE. We are now addressing the problem of this apparent heterogeneity and the taxonomic signification of genome structure.

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REFERENCES

1. **Allardet-Servent, A., M. J. Carles-Nurit, G. Bourg, S. Michaux, and M. Ramuz.** 1991. Physical map of the *Brucella melitensis* 16M chromosome. J. Bacteriol. **173:**2219–2224.
2. **Allardet-Servent, A., S. Michaux-Charachon, and M. Ramuz.** 1993. *Agrobacterium tumefaciens* C58 contains one circular and one linear chromosome. Presented at the First International Symposium Mapping and Sequencing of Small Genomes, 28 to 30 March 1993, Institut Pasteur, Paris, France.
3. **Barbour, A., and S. F. Hayes.** 1986. Biology of *Borrelia* species. Microbiol. Rev. **50:**381–400.
4. **Baril, C., C. Richaud, G. Baranton, and I. Saint Girons.** 1989.

Linear chromosome of *Borrelia burgdorferi*. Res. Microbiol. **140:** 507–516.
5. **Burkhardt, B., D. Schillik, and A. Puhler.** 1987. Physical characterization of *Rhizobium meliloti* megaplasmids. Plasmid **17:**13–25.
6. **Crespi, M. E., Messens, A. B. Caplan, M. van Montagu, and J. Desomer.** 1992. Fasciation induction by the phytopathogen *Rhodococcus fascians* depends upon a linear plasmid encoding a cytokinin synthase gene. EMBO J. **11:**795–804.
7. **Davidson, B. E., J. MacDougall, and I. Saint Girons.** 1992. Physical map of the linear chromosome of the bacterium *Borrelia burgdorferi* 212, a causative agent of Lyme disease, and localization of rRNA genes. J. Bacteriol. **174:**3766–3774.
8. **Douglas, C. J., R. J. Staneloni, R. A. Rubin, and E. W. Nester.** 1985. Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. J. Bacteriol. **161:**850–860.
9. **Ferdows, M. S., and A. G. Barbour.** 1989. Megabase-sized linear DNA in the bacterium *Borrelia burgdorferi*, the Lyme disease agent. Proc. Natl. Acad. Sci. USA **86:**5969–5973.
10. **Fonstein, M., S. Zengh, and R. Haselkorn.** 1992. Physical map of the genome of *Rhodobacter capsulatus* SB 1003. J. Bacteriol. **174:**4070–4077.
11. **Frothingham, R., R. L. Allen, and K. H. Wilson.** 1991. Rapid 16S ribosomal DNA sequencing from a single colony without DNA extraction or purification. BioTechniques **11:**40–44.
12. **Herdman, M.** 1985. The evolution of bacterial genomes, p. 37–68. *In* T. Cavalier-Smith (ed.), The evolution of genome size. John Wiley & Sons, Inc., New York.
13. **Hinnebush, J., and A. G. Barbour.** 1991. Linear plasmids of *Borrelia burgdorferi* have a telomeric structure and sequence similar to those of a eukaryotic virus. J. Bacteriol. **173:**7233–7239.
14. **Hooykaas, P. J. J., R. Peerbolte, A. J. G. Regenburg-Tu¨ink, P. de Vries, and R. A. Schilperoot.** 1982. A chromosomal linkage map of *Agrobacterium tumefaciens* and comparison with the maps of *Rhizobium* spp. Mol. Gen. Genet. **188:**12–17.
15. **Hynes, M. F., R. Simon, and A. Pühler.** 1985. The development of plasmid-free strains of *Agrobacterium tumefaciens* by using incompatibility with a *Rhizobium meliloti* plasmid to eliminate pAtC58. Plasmid **13:**99–105.
16. **Jordan, D. C.** 1984. Family III. *Rhizobiaceae* Conn 1938, 321^{AL}, p. 234–256. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
17. **Krawiec, S., and M. Riley.** 1990. Organization of the bacterial chromosome. Microbiol. Rev. **54:**502–539.
18. **Kündig, C., H. Hennecke, and M. Göttfert.** 1993. Correlated physical and genetic map of the *Bradyrhizobium japonicum* 110 genome. J. Bacteriol. **175:**613–622.
19. **Leblond, P., M. Redenbach, and J. Cullum.** 1993. Physical map of the *Streptomyces lividans* 66 genome and comparison with that of the related strain *Streptomyces coelicolor* A3(2). J. Bacteriol. **175:**3422–3429.
20. **Levene, S. D., and B. H. Zimm.** 1987. Separation of open circular DNA using pulsed-field electrophoresis. Proc. Natl. Acad. Sci. USA **84:**4054–4057.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Michaux, S., J. Paillisson, M.-J. Carles-Nurit, G. Bourg, A. Allardet-Servent, and M. Ramuz.** 1993. Presence of two independent chromosomes in the *Brucella melitensis* 16M genome. J. Bacteriol. **175:**701–705.
23. **Miller, I. S., D. Fox, N. Saeed, P. A. Borland, C. A. Miles, and G. R. K. Sastry.** 1986. Enlarged map of *Agrobacterium tumefaciens* C58 and the location of chromosomal regions which affect tumorigenicity. Mol. Gen. Genet. **205:**153–159.
24. **Moreno, E., E. Stackebrandt, M. Dorsh, J. Wolters, M. Bush, and H. Mayer.** 1990. *Brucella abortus* 16S rRNA and lipid A reveal a phylogenetic relationship with members of the alpha-2 subdivision of the class *Proteobacteria*. J. Bacteriol. **172:**3569–3576.
25. **Old, I. G., J. MacDougall, I. Saint Girons, and B. E. Davidson.** 1992. Mapping of genes on the linear chromosome of the bacterium *Borrelia burgdorferi*: possible locations for its origin of replication. FEMS Microbiol. Lett. **99:**245–250.
26. **Old, I. G., D. Margarita, and I. Saint Girons.** 1993. Unique genetic arrangement in the *dnaA* region of *Borrelia burgdorferi* linear chromosome: nucleotide sequence of the *dnaA* gene. FEMS Microbiol. Lett. **111:**109–114.
27. **Saint Girons, I., S. J. Norris, U. Göbel, J. Meyer, E. M. Walker, and R. Zuerner.** 1992. Genome structure of spirochetes. Res. Microbiol. **143:**615–621.
28. **Sobral, B. W. S., R. J. Honeycutt, A. G. Atherly, and M. McClelland.** 1991. Electrophoretic separation of the three *Rhizobium meliloti* replicons. J. Bacteriol. **173:**5173–5180.
29. **Suwanto, A., and S. Kaplan.** 1989. Physical and genetic mapping of the *Rhodobacter sphaeroides* 2.4.1. genome: presence of two unique circular chromosomes. J. Bacteriol. **171:**5850–5859.
30. **Suwanto, A., and S. Kaplan.** 1992. Chromosome transfer in *Rhodobacter sphaeroides*: Hfr formation and genetic evidence for two unique circular chromosomes. J. Bacteriol. **174:**1135–1145.
31. **Van Larebeke, N., G. Engler, M. Holsters, S. Van den Elsacher, I. Zaenen, R. A. Schilperoort, and J. Schell.** 1974. Large plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing ability. Nature (London) **242:**171–172.
32. **Wabiko, H.** 1992. Sequence analysis of an insertion element, IS1131, isolated from the nopaline-type Ti plasmid of *Agrobacterium tumefaciens*. Gene **114:**229–233.
33. **Walker, E. M., J. K. Arnett, J. D. Heath, and S. J. Norris.** 1991. *Treponema pallidum* subsp. *pallidum* has a single, circular chromosome with a size of ~900 kilobase pairs. Infect. Immun. **59:**2476–2479.
34. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.
35. **Wheelis, M. L., O. Kandler, and C. R. Woese.** 1992. On the nature of global classification. Proc. Natl. Acad. Sci. USA **89:**2930–2934.
36. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.
37. **Yanagi, M., and K. Yamasato.** 1993. Phylogenetic analysis of the family *Rhizobiaceae* and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. FEMS Microbiol. Lett. **107:**115–120.